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Lipid Nanoparticles for Gene Delivery

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Abstract

Nonviral vectors which offer a safer and versatile alternative to viral vectors have been developed to overcome problems caused by viral carriers. However, their transfection efficacy or level of expression is substantially lower than viral vectors. Among various nonviral gene vectors, lipid nanoparticles are an ideal platform for the incorporation of safety and efficacy into a single delivery system. In this chapter, we highlight current lipidic vectors that have been developed for gene therapy of tumors and other diseases. The pharmacokinetic, toxic behaviors and clinic trials of some successful lipids particles are also presented.

1. INTRODUCTION

Lipid nanoparticles (LNPs) have been developed and used extensively as nonviral (or synthetic) vectors to treat genetic and acquired disorders in gene therapy. LNPs are safer than viral vectors due to the absence of immunogenic viral proteins. LNPs have shown robust capability to condense and deliver various nuclei acid molecules ranging in size from several nucleotides (RNA) to several million nucleotides (chromosomes) to cells (Figure 2.1). LNPs are also easy to scale up due to established construction protocols and can be easily modified by the incorporation of targeting ligands. In general, there are three major ways to develop lipidic vectors for suitable gene transfection. The first approach is to screen libraries of lipids to select the most effective structure and biocompatible material for various applications. For example, in the study by Anderson et al., numerous lipids of different structures from the lipid library have been successfully selected and developed to improve the therapeutic efficacy for the treatment of various acute and chronic diseases (Chen et al., 2012; Dong et al., 2014; Whitehead et al., 2014). More details are described in the following chapter of this book by Anderson et al. A second approach is to modify current existing lipid materials to enhance the therapeutic efficacy. Some of them have emerged as promising approaches in clinical trials (Tabernero et al., 2013). A third approach is to develop the new materials to deliver genetic material to the target cells (Koynova & Tenchov, 2011). The barriers of gene expression will be briefly described. Several novel lipids and strategies for the improved delivery of nucleic acids are reviewed with an emphasis on the methods of overcoming the limitations caused by the barriers. In addition, we highlight applications of LNP gene therapy in several diseases. Furthermore, the latest studies of pharmacokinetics, biodistribution, and toxicity of LNP gene therapy will be

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included. In the end, promising clinical studies of LNP-based gene therapy will be discussed.

2. RATIONAL DESIGNS TO OVERCOME EXTRACELLULAR AND INTRACELLULAR BARRIERS

Many disorders, such as cancer, are disseminated and widespread throughout the body, thus intravenous injection of agents is the most common, but also the most complex, route in gene therapy. From the moment of injection until the agent reaches targeted cells, genetic material encounters extracellular and intracellular barriers that affect the therapeutic results. First, naked RNAs or DNAs are unstable under physiological conditions, resulting in enzymatic degradation by endogenous nucleases and clearance by the reticuloendothelial system (RES). Second, RNAs or DNAs are anionic hydrophilic polymers that are not favorable for uptake by cells, which are also anionic at the surface. Third, the off-target effect of genes will lead to unwanted toxicities in normal tissues. Furthermore, immune stimulation upon injection hinders further development of new gene therapies. The success of gene therapy depends largely on the development of a vehicle or vector that can efficiently and effectively deliver genetic material to target cells and obtain sufficient levels of gene expression in vivo with minimal toxicity. Virus-derived vectors for gene therapy are efficient in gene delivery and transfer, but safety issues limit the use of viral vectors in gene therapy. To date, the rational designs of nonviral vectors have been focused on overcoming the extracellular and intracellular barriers in the delivery of genetic material to targeted cells.

2.1 Extracellular Barriers

Once exogenous genes enter the human biological system, they are recognized by the RES as foreign pathogens and cleared from blood circulation before having the chance to encounter target cells within or outside the vascular system (Mastrobattista, van der Aa, Hennink, & Crommelin, 2006). It has been reported that the half-life of naked DNA in the blood stream is around several minutes (Kawabata, Takakura, & Hashida, 1995). Upon injection, DNA was rapidly degraded by enzymes and eliminated from plasma due to extensive uptake by the liver (Kawabata et al., 1995). Chemical modification and a proper delivery method can reduce uptake by RES and protect nucleic acids from degradation by ubiquitous nucleases, which increase stability and efficacy of gene therapy.

Many efforts have been made to increase the stability and half-life of liposomes in the body by incorporation of helper components. For example, Damen (Damen, Regts, & Scherphof, 1981) and Semple (Semple, Chonn, & Cullis, 1996) incorporated cholesterol into the membrane to reduce the mobility of phospholipid molecules and increase packing of phospholipid.

Coating the liposome with polyethylene glycol (PEG), or PEGylation, is typically the method used to protect nanoparticles from the immune system and escape RES uptake (Jokerst, Lobovkina, Zare, & Gambhir, 2011). Since 1990s, PEGylation has been widely used to stabilize liposomes and their payloads through physical, chemical, and biological mechanisms. Detergent-like PEG lipids (e.g., PEG-DSPE) can enter the liposome to form a

hydrated layer and steric barrier on the liposome surface. Based on the degree of PEGylation, the surface layer can be generally divided into two types: brush-like and mushroom-like layers. For PEG-DSPE-stabilized liposomes, PEG will take on the mushroom conformation at a low degree of PEGylation (usually less than 5 mol%) and will shift to brush conformation as the content of PEG-DSPE is increased past a certain level (Guo & Huang, 2011). It has been shown that increased PEGylation leads to a significant increase in the circulation half-life of liposomes (Huang & Liu, 2011; Li & Huang, 2010). However, due to the detergent-like property of PEG-DSPE, the brush layer with high PEGylation degree is not stable. Li and Huang discovered that PEGylated liposome–polycation–DNA (LPD) nanoparticles overcome this issue (Li & Huang, 2010). The LPD nanoparticle is stabilized by electrostatic interactions within the negatively charged nucleic acid–protamine complex core and positively charged lipid bilayer. This core-surface type of liposome was able to support the bilayer and tolerate a high level of PEG-DSPE (10 mol%) with a relatively dense PEG brush structure on the surface. Most importantly, these liposomes were not taken up by the liver Kupffer cells (Li & Huang, 2009). Furthermore, modification of sheddable PEG with tumor-specific ligands or pH-sensitive linkers has extended the use of LNPs in gene therapy. However, upon multiple injections, PEGylated LNP loses its ability to circulate for long periods in the bloodstream, a phenomenon known as accelerated blood clearance (ABC) (Dams et al., 2000; Gomes-da-Silva et al., 2012). The mechanism of ABC is associated with activation of anti-PEG-specific IgM after the first dose of PEGylated liposome (Ishida et al., 2006).

Recently, Liu, Hu, and Huang (2014) used the lipid bilayer core structure of the lipid–calcium–phosphate (LCP) NPs to examine the effects of the density of PEG and the incorporation of various lipids onto the surface in vivo. In their study, they demonstrated that delivery to hepatocytes was dependent on both the concentration of PEG and the surface lipids. Moreover, LCP NPs could be directed from hepatocytes to Kupffer cells by decreasing PEG concentration on the particle surface. Positively charged lipid 1,2-dioleoyl-3-trimethylammonium-propane exhibited higher accumulation in the hepatocytes than LCP NPs with neutral lipid dioleoylphosphatidylcholine.

As a systemic delivery carrier, LNPs must be stable enough to remain in circulation for an extended period and accumulate at disease sites via the enhanced permeability and retention (EPR) effect. In addition to working with lipid vectors, recent studies have also found that chemically modified nucleic acids can increase stability by altering the physicochemical properties. For instance, without significant loss of RNA interference activity, Czauderna et al. showed that chemical modification of siRNA at different positions can stabilize siRNA against serum-derived nucleases and prolong the circulation time in the blood (Czauderna et al., 2003).

2.2 Intracellular Barriers

It has been reported that although >95% of cells in culture typically internalize vectors, only a small fraction, typically <50%, express the transgene (Mark, 2003). Following internalization, gene delivery vectors are challenged by intracellular barriers, including endosome entrapment, lysosomal degradation, nucleic acid unpacking from vectors,

translocation across the nuclear membrane (for DNA), release at the cytoplasm (for RNA), and so on. Successful gene therapy depends upon the ability of the vector to deliver the nucleic acids to the target sites inside of the cells in order to obtain sufficient levels of gene expression. The relative contribution of distinct endocytic pathways, including clathrin- and caveolae-mediated endocytosis and/or macropinocytosis, is not yet well defined. Escape of DNA/RNA from endosomal compartments is thought to represent a major obstacle. LNPs have shown the unique ability to deliver nucleic acids by endosomal escape. Initially, Szoka et al. proposed that anionic phospholipids could displace cationic lipids from plasmids, thus assisting the release of plasmid following uptake of the complex into cells (Xu & Szoka, 1996; Zelphati & Szoka, 1996). It is also suggested that cationic lipids form ion pairs with anionic lipids within the endosome membrane leading to disruption of the endosomal membrane following uptake of nucleic acid–cationic lipid complexes into cells. This facilitates cytoplasmic release of the plasmid or oligonucleotide (Hafez, Maurer, & Cullis, 2001). In addition, Cullis et al. proposed that mixtures of cationic lipids and anionic phospholipids preferentially adopt the inverted hexagonal (H_{II}) phase, therefore facilitating escape of the plasmid from the endosome into the cytoplasm (Cullis, Hope, &Tilcock, 1986; Hafez et al., 2001). Significant intracellular hurdles beyond endosomal escape include the limited nuclear entry (Brunner et al., 2000; Dean, Strong, & Zimmer, 2005) and inefficient intranuclear release of plasmid for transcription (Hama et al., 2006). The transfection efficiency of nanoparticles is also related to the cell cycle and is enhanced by mitotic activity. For instance, Brunner's study showed that the high transfection close to the M phase is facilitated perhaps by nuclear membrane breakdown at this phase (Brunner et al., 2000). Hama et al. compared the intracellular trafficking and nuclear transcription between adenoviral and lipoplex (lipofectamine plus). In their observation, although lipoplex system has higher cellular uptake than that of adenoviral vector, the nuclear transfer efficiency of lipoplex is found to be lower than the adenoviral one, suggesting that the difference in transfection efficiency principally arises from differences in nuclear transcription efficiency and not from a difference in intracellular trafficking (Hama et al., 2006).

3. CURRENT LIPIDIC VECTORS FOR GENE DELIVERY

3.1 Cationic Lipids

Cationic lipids were introduced as carriers for delivery of nucleic acids for gene therapy over two decades ago (Malone, Felgner, & Verma, 1989; Schroeder, Levins, Cortez, Langer, & Anderson, 2010). They are still the major carriers for gene delivery, because they can be easily synthesized and extensively facilitated by modifying each of their constituent domains. Cationic lipids can be used as vectors to condense and deliver anionic nucleic acids through electrostatic interactions. These nanostructured complexes, called "lipoplexes," have shown to be extremely useful vehicles in gene therapy. By modulating the ratio of cationic lipids and nucleic acids, the excess cationic coating was able to facilitate the binding of vectors with negatively charged cell surfaces, and furthermore interruption with endosomal membrane to help cytoplasmic delivery of nucleic acids. However, lipoplex suspensions are known to be unstable in aqueous suspension for long-term storage, especially with respect to hydrolysis and size stability (Fehring et al., 2014). DNA can be encapsulated in liposomal

formulations by thin film, reverse-phase evaporation and asymmetric liposome formation methods (Levine, Pearce, Adil, & Kokkoli, 2013).

It has been demonstrated that the physicochemical properties of cationic lipids significantly limit the cellular uptake and transfection efficiency in gene therapy. In a study by Ross et al, it was found that the size of the lipoplex is a major determinant of in vitro lipofection efficiency (Ross & Hui, 1999). Furthermore, reports from different laboratories have demonstrated that larger liposomes are eliminated from the blood circulation more rapidly than smaller ones (Senior, 1987) and positively charged liposomes have a shorter half-life than neutral or negative ones (Immordino, Dosio, & Cattel, 2006).

Following previous fundamental studies on the structure–activity relationship of cationic lipids, it is well accepted that the polar headgroup, hydrophobic moiety, and linker are three important constituent domains for cationic lipids. While hydrophobic regions, including the length and the degrees of nonsaturation of the alkyl chains, are relatively similar, the structure and component of polar headgroup and linkers are substantially different. The polar hydrophilic headgroup is positively charged, usually through the protonation of one or several amino groups. They can be quaternary ammoniums, amines, amino acids or peptides, guanidiniums, heterocyclic headgroups, and some unusual headgroups (Zhi et al., 2013). The hydrophobic portion of lipid is composed of a steroid or alkyl chains (saturated or unsaturated). The headgroups of cationic lipids exhibiting one or more positive charges can condense negatively charged nucleic acids through electrostatic attraction. This binding force plays an important role in the therapeutic efficacy of gene therapy. On one hand, it has to be strong enough to protect nucleic acids from degradation during circulation and transportation. On the other hand, it also has to be weak enough to allow for timely release of the payload of nucleic acids within target cells.

Semple et al. found that the acid dissociation constant (pKa) of the head-group and the distance of the charge presented to the lipid bilayer interface are the most important parameters for siRNA delivery in vivo (Semple et al., 2010). Recently, Alabi et al. also supported the observation that they found the pKa of LNPs showed the strongest correlation with biological barriers and gene silencing (Alabi et al., 2013). The presence of a charge on the lipid can lead to toxic side effects and rapid clearance from the circulation. To address this problem, anionic lipids with pKa values of 7 and lower have been synthesized, which have presented lower toxicity and efficient encapsulation of nucleic acids for both in vitro and in vivo activity (Tam, Chen, & Cullis, 2013). More details about LNPs for short interfering RNA delivery are presented in the following chapter of this book by Cullis et al.. When vectors reach the physiological acidic environment of the endosome, the amine should be protonated and become positively charged, associating with the anionic endosomal lipids, inducing an endosomolytic H_{II} (inverted micelle) phase structure. This interaction will induce the destabilization of the endosomal membranes and promote the release of siRNA into the cytosol. Schroeder et al. had shown that molecules containing several amines per head group are able to adhere to the negatively charged siRNA in a better manner than several lipids containing a single positive charge per headgroup (Schroeder et al., 2010).

3.2 Ionizable Lipids and Lipidoids

Ionizable lipids are an advanced delivery platform of gene therapy that can self-assemble into nanoparticles when mixed with polyanionic nucleic acids. Ionizable cationic lipids with modulated pKa values increase nucleic acid payload and enhance the therapeutic efficacy of gene therapy. At formulating step, where there is a low pH condition, ionizable lipids will become positive charged, resulting in high nucleic acids loading. While, upon injection, in physiological environments where the pH is above the pKa of the ionizable lipids, the surface of the LNPs has an almost neutral charge that can evade RES uptake, improve circulation, and reduce toxicity (Tam et al., 2013). However, once nanoparticles are internalized into the endosome, where the pH is lower than the pKa of the lipids, the amino group of the ionizable lipid becomes protonated and associates with the anionic endosomal lipids, which facilitate endosome escape. Recently, two promising ionizable cationic lipids (Figure 2.2), DLin-KC2-DMA (2,2-dilin-oleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane) with a pKa of 6.7, and DLin-MC3-DMA (1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane) with a pKa of 6.4, have been successfully developed to formulate stable nucleic acid lipid particles (SNALPs) (Heyes, Palmer, Bremner, & MacLachlan, 2005; Jayaraman et al., 2012; Semple et al., 2010), which are 100-fold and 1000-fold more potent in silencing hepatic genes in comparison to the previously used lipids (Heyes et al., 2005). Most excitingly, they decreased the half-maximal effective dose (ED_{50}) of the siRNA in rodents from ~0.1 mg/kg to ~0.02 mg/kg and presented excellent silencing activity in rodents as well as nonhuman primates. Recently, Tekmira, a biopharmaceutical company, announced in its website that the third generation lipids successfully integrated to deliver mRNA, and achieved a higher efficacy than DLin-MC3-DMA LNPs, however, the details about this lipid is not yet published.

A new class of lipid-like material, termed lipidoids, which contain tertiary amines, are one of the most innovative and promising nonviral lipid vehicles for RNAi therapeutics (Akinc et al., 2008). They are prepared by conjugating commercially available amines (Figure 2.2.) (Akinc et al., 2009; Love et al., 2010; Sun et al., 2012). Notably, the synthesis reaction for generating a lipidoid library proceeds in the absence of solvent or catalysts, and thereby eliminates the purification or concentration steps (Akinc et al., 2008). Lipidoids and lipids share many of the physicochemical properties that drive the formation of liposomes for gene delivery. However, lipidoids are easy to synthesize and purify and do not require a colipid for efficient DNA delivery. These advantages make high-throughput combinatorial synthesis of lipidoids possible and allow for rapid in vitro screening of thousands of potential drug delivery candidates (Figure 2.2). By varying the types of amines and the lengths and types (acrylamide/acrylate/epoxide) of tails, Sun et al. were able to build a structurally diverse library (Sun et al., 2012). Lipidoids will be discussed in further detail in the following chapter of this book by Anderson et al.

3.3 Gene-Lipid Conjugates

As known, nucleic acids are rapidly degraded in serum or inside cells and must be protected from nuclease attack. Even though cationic lipids with different functionalities have been used to encapsulate nucleic acids from degradation and enhanced the therapeutic efficacy, several studies have shown that cationic lipids exhibit severe toxicities, resulting in the

limitation of further clinical applications (Soenen, Brisson, & De Cuyper, 2009; Yew & Scheule, 2005). The simplest approach to increase nuclease stability is to directly modify the internucleotide phosphate linkage (Behlke, 2008). Instead of providing a carrier for nucleic acids, several studies have reported that nucleic acid conjugation could improve in vivo pharmacokinetic behavior of genetic materials, providing an alternative approach for gene therapy (Chillemi, Greco, Nicoletti, & Sciuto, 2013; Koppelhus, Shiraishi, Zachar, Pankratova, & Nielsen, 2008; Kubo et al., 2013). Conjugating the lipids to the site of nucleic acids without loss of bioactivity is the key step for modification. Replacement of a nonbridging oxygen with sulfur, boron, nitrogen, or methyl groups provides nuclease resistance and has been extensively explored for use in antisense applications (Behlke, 2008). Exogenous siRNA can activate the innate immune system through toll-like receptors (TLR), but introduction of 2′-O-methyl (2′OMe) to nucleotide can inhibit the TLR-associated inflammatory response (Judge, Bola, Lee, & MacLachlan, 2006).

Hydrophobic lipids can also be attached to siRNAs to change the biodistribution, extend circulation time, and facilitate direct cellular uptake (Lorenz, Hadwiger, John, Vornlocher, & Unverzagt, 2004; Soutschek et al., 2004; Wolfrum et al., 2007). For example, cholesterols have been successfully introduced to conjugate to the 3′-terminus of the sense strand of siRNA nucleic acids for systemic delivery via a pyrrolidone linkage (Soutschek et al., 2004). The conjugate (chol-siRNA) exhibited increased cellular transfer efficiency and improved in vivo pharmacokinetic behaviors without a significant loss in silencing ability. Another biocompatible material, α-tocopherol (vitamin E), can be covalently conjugated to the 5′-terminus of the antisense strand of siRNA to achieve a significant reduction in targeted protein without induction of inflammatory interferon (Nishina et al., 2008). However, chemical modification of siRNA alone often results in renal clearance of intact siRNA without degradation (Behlke, 2008). As such, for future application of chemically modified siRNA, rational design and increased specificity are in great need.

3.4 LNP Functionalization

To enhance targeted delivery, several functional LNPs for gene therapy have been developed recently. With these proof-of-concept systems, functionalized particles efficiently delivered associated nucleic acids to the targeted cells. The first strategy is to modify the nanoparticles with tumor-specific ligand to enhance intracellular uptake. For example, iron-saturated transferrin (Tf) (Huang et al., 2013), folic acid (Hu et al., 2014; Xiang et al., 2013), RGD (Han et al., 2010; Majzoub et al., 2014), and anisamide (Li, Chono, & Huang, 2008) have been widely applied for specific gene delivery.

The rational design of LNPs to escape from endosomal/lysosomal vesicles is another strategy to enhance the efficacy of gene therapy. The extra-cellular pH of tumor inflammatory tissues is lower than other physiological tissues. Following internalization, most vectors end up in compartments with a lower pH. Endosomes have a pH around 5.5–6.0 and lysosomes about 4.5. Thus, if pH-sensitive functional groups are applied to the LNPs, they may become protonated in the low-pH environment. This would result in lower toxicity and facilitate the delivery of nucleic acids before degradation (Hu et al., 2014). Generally, pH-sensitive lipids contain a tertiary amine instead of a quaternary ammonium

group, which results in a cationic charge at an acidic pH and almost neutral at physiological pH (Sato et al., 2012). Moreover, there are some successful pH-sensitive linkers applied to nanoparticles to achieve more specific delivery, for example, diorthoester, orthoester, vinyl ether, phosphoramidate, hydrazine, and beta-thiopropionate (Romberg, Hennink, & Storm, 2008).

Magnetic LNPs are particles that have magnetic cores with a lipid coating that can be functionalized by attaching therapeutic nucleic acids to correct a genetic defect (McBain, Yiu, & Dobson, 2008; Ranjan & Kinnunen, 2012). Biocompatible and biodegradable iron oxide nanoparticles can be used as contrast enhancement agents for magnetic resonance imaging and also act as effective carriers for genes (Jiang, Eltoukhy, Love, Langer, & Anderson, 2013; McBain, Yiu, & Dobson, 2008). Jiang et al. used C14–200 lipidoids and DSPC to coat iron oxide nanoparticles in N-methyl-2- pyrrolidone solvent and showed efficient DNA and siRNA delivery upon the application of an external magnetic field, with performance exceeding that of Lipofectamine 2000 (Jiang et al., 2013). Kenny et al. developed an MRI-visible gene delivery nanocomplex system comprised of self-assembling mixtures of liposomes, plasmid DNA, and targeting ligands, which specifically enhanced transfection efficiency and allowed real-time in vivo monitoring of the specific tumor tissue (Kenny et al., 2012). In another study, Writer et al. prepared lipid peptide nanocomplexes with Gadolinium-chelated lipid, DNA-binding peptide, and plasmid DNA (Writer et al., 2012). These lipid nanocomplexes can be used for gene delivery and MRI imaging in the brain. LipoMag, a novel LNP developed by Namiki et al., is made of an oleic acid-coated magnetic nanocrystal core and a cationic lipid shell (Namiki et al., 2009). It displayed efficient gene silencing and antitumor efficacy without an adverse immune reaction upon injection in mice bearing gastric tumors.

Microbubble ultrasound contrast agents have the potential to dramatically improve gene therapy treatments by enhancing the delivery of therapeutic nucleic acids to malignant tissues. Ultrasound technology has the ability to improve cell membrane permeability, modulate vascular permeability, and enhance endocytic uptake in cells. In a recent study by Fujii et al., ultrasound-mediated transfection of VEGFR2 shRNA plasmid-bearing microbubbles resulted in knockdown of VEGFR2, leading to an antiangiogenic effect and reduced tumor growth (Fujii et al., 2013). Song et al. explored high-intensity therapeutic ultrasound- and microbubble-mediated gene delivery. Maximum gene expression in treated animals was 700-fold greater than in negative controls (Song, Shen, Chen, Brayman, & Miao, 2011).

4. GENE THERAPY APPLICATIONS

Up until 2014, over 2000 clinical trials, comprised of virtually all types of human disorders, have been conducted or were currently ongoing for gene therapy. The number of clinical trials is still increasing due to the promising opportunity to correct gene disorders.

4.1 Gene Therapy for Cancer

Much attention of today’s cancer research is focused on finding missing or defective genes that cause or increase an individual’s risk of certain types of cancer. Over 60% of the gene

therapy clinical trials conducted have been in the field of cancer (Giacca, 2010). Cancer gene therapy can benefit from two aspects based on the mechanism of gene medicines. First, gene therapy can directly affect specific genes that cause cancer at the molecular level. Second, gene therapy can prevent cancer by improving the immune system through identifying the susceptibility genes. In other words, LNP-based cancer gene therapy can follow two alternative approaches: eliminate the cancer cells or improve the efficacy of the immune system by recognizing and destroying cancer cells.

As a result of rapid, defective angiogenesis, tumor blood vessels are highly permeable, leading to accumulation of nanoparticles at the tumor site. Furthermore, tumors are characterized by dysfunctional lymphatic drainage that extends the retention of LNPs at the tumor site. This behavior of nanoparticles is called the EPR effect proposed by Dr Maeda (Maeda, 2012; Maeda, Nakamura, & Fang, 2013; Matsumura & Maeda, 1986). However, the leakage of blood vessels in different types of tumors is quite different and limited experimental data from patients on the effectiveness of this mechanism have hindered the development of effective drugs (Prabhakar et al., 2013). Vascular permeability is the key factor involved in the EPR effect in cancer. It is well accepted that vascular endothelial growth factor (VEGF) enhances the vascular permeability of tumor vessels. In a recent study by Zhang, Schwerbrock, Rogers, Kim, and Huang (2013), VEGF siRNA and gemcitabine monophosphate (GMP) were encapsulated into a single cell-specific, targeted LCP nanoparticle formulation, resulting in 30–40% induction of tumor cell apoptosis, eightfold reduction of tumor cell proliferation, and significant decrease of tumor microvessel density. This combination therapy led to improved therapeutic response in comparison to either VEGF siRNA or GMP therapy alone. Recently, first-in-humans trial of an RNAi therapy targeting VEGF and kinesin spindle protein in cancer patients was performed using LNP-formulated siRNA therapy (Tabernero et al., 2013). They detected the drug in tumor biopsies, siRNA-mediated mRNA cleavage in the liver, downregulation of the targeted gene, and antitumor activity. These results presented proof-of-concept for RNAi therapeutics with LNP formulation in humans.

4.2 Gene Therapy in Liver Disease

Liver diseases, including inherited metabolic disorders, chronic viral hepatitis, liver cirrhosis, and primary and metastatic liver cancer constitute a formidable health problem due to their high prevalence and the limitations of current therapies (Domvri et al., 2012; Gonzalez-Aseguinolaza & Prieto, 2011; Prieto et al., 2004). For most of the inherited metabolic liver diseases, no effective therapy is currently available other than liver transplantation, which is hampered by donor shortage, cost, surgical risks, and long-term immunosuppression. Thus, safer and more efficient therapies are greatly needed. Nonviral carriers for liver gene therapy can fulfill these needs as they are able to delivery gene-based medicines more specifically to the liver with minimized toxicity and immunogenicity. Taking advantage of special membrane receptors located on liver cell membrane, nonviral vectors, especially LNPs, can be modified with targeting moieties and deliver the targeted genes to liver. Several attempts have shown potential success in liver disease. For example, collagen type VI receptor (Du et al., 2007), mannose-6-phosphate receptor (Adrian et al., 2007), and galactose receptor (Mandal, Das, Basu, Chakrabarti, & Das, 2007) have been

successfully targeted. Sato et al. designed vitamin A-coupled liposome to deliver siRNA for liver cirrhosis. In their study, only five treatments with the collagen-specific liposomes almost completely resolved liver fibrosis and prolonged survival in rats with otherwise lethal dimethylnitrosamine-induced liver cirrhosis in a dose- and duration-dependent manner (Sato et al., 2008).

5. PHARMACOKINETICS, BIODISTRIBUTION AND TOXICITY OF LNPS

5.1 Pharmacokinetics and Biodistribution Profile of LNPs

It is well known that the systemic delivery of naked DNA or siRNA alone often lead to fast clearance and degradation. A variety of lipid vectors hold the potential to improve gene therapy. As long as the nucleic acids are completely encapsulated in stable vectors, the system could provide protection to the nucleic acids from degradation, and thus, the vector will be able to represent the biodistribution of whole system. One of the key reasons for this success is that LNPs are able to provide better biodistribution and pharmacokinetics profiles of genes in vivo.

In order to study LNPs, the nucleic acids and vehicles were labeled with radioactive isotopes and tracked upon the administration. Replacing ^{1}H or ^{12}C atoms of nucleic acids with radioactive ^{3}H or ^{14}C does not alter the structure of nucleic acid and have the least impact on the pharmacokinetic behavior of nucleic acid itself. However, van de Water et al. reported a head-to-head comparison of ^{3}H- versus ^{111}In-labeled unformatted siRNA, and found that they have different distributions and pharmacokinetics (Christensen et al., 2013; van de Water et al., 2006). Therefore, it is important to choose the right modification of radioactive isotopes to monitor the behavior of nanoparticles in the body. However, as radioactive compounds are potential health hazards some studies use fluorescence imaging to track the distributions and pharmacokinetic profiles of LNPs in gene therapy. Although fluorescent dyes are relatively safe with low cost, they may not be the best option. For example, Liu et al. recently compared radioactive isotopes and fluorescence imaging using LCP nanoparticles and found that while radioactive isotopes showed the liver and spleen as the major accumulation sites, fluorescence imaging indicated tumor accumulation was predominant. A possible explanation for this difference is that the liver and spleen have strong intrinsic tissue absorption and light scattering which quenches fluorescence (Liu, Tseng, & Huang, 2012).

In general, following systemic injection, positively charged lipid/nucleic acid formulation will bind to various types of serum proteins such as albumin, heparin, lipoprotein, specific opsonins, and others. The binding force is dependent on net charge density and surface morphology of the lipid/plasmid complex (Thierry et al., 1997). Extensive lung accumulation was observed after injection, which may be the result of entrapment of complexes in lung capillaries by the first-passage effect. Lung deposition may also be due to ionic association with the large surface area of the lung endothelia (Mahato et al., 1998). Negatively charged complexes did not show lung accumulation (Ishiwata, Suzuki, Ando, Kikuchi, & Kitagawa, 2000).

5.2 Toxicity of LNPs

The composition of LNPs for the gene therapy can be divided into two parts: nucleic acids and lipids. Thus, safety issues related to LNPs in gene therapy are caused by nucleic acid- and lipid-mediated side effects. The major problem in gene therapy is the off-targeting effect, in which nucleic acids will distribute nonspecifically throughout the whole body. Studies have shown that introduction of dsRNA longer than 29–30 bp into mammalian cells results in a potent activation of interferon response and have precluded its use in RNAi-based therapy (Huang & Liu, 2011). Exogenous siRNA can activate the innate immune system through TLR 7 and 8. Thus, careful design and adequate control are greatly needed for gene therapy if naked nucleic acids are directly administrated into the body.

An off-target side effect of naked nucleic acids can be partially eliminated through incorporation into a lipid formulation conjugated with a targeting moiety. However, some lipids are also immunogenic. Of these lipids, the immunostimulating effects are reported to be stronger in cationic liposomes than anionic or neutral liposomes. Cationic liposomes alone can stimulate antigen-presenting dendritic cells leading to the expression of co-stimulatory molecules, CD80 and CD86 (Vangasseri et al., 2006). Recently, Omidi et al. used a microarray method to evaluate the toxicogenomics and genotoxic potential in a biological system after cationic lipid-based gene therapy (Omidi, Barar, & Akhtar, 2005) and found that cationic lipid Oligofectamine nanosystems in human alveolar epithelial A549 cells induced significant gene expression changes belonging to the different genomic ontologies such as cell defense and apoptosis pathways (Omidi et al., 2008). The data indicated the importance of safety and immunogenicity examination of new lipids for gene therapy.

6. CLINICAL TRIALS

Academic and industrial researchers have made steady progress in gene therapy since Friedmann et al. first proposed the use of genes for human genetic disease in 1972 (Friedmann & Roblin, 1972). Recently, several therapeutic agents targeting various types of diseases have reached different stages of clinical trials (Table 2.1). Alnylam Pharmaceuticals are developing aggressive LNP therapeutic agents using Enhanced Stabilization Chemistry-GalNAc-conjugate delivery technology. For example, ALN-TTRsc (targeting TTR for treatment of transthyretin-mediated amyloidosis) and ALN-PCS02 (targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) to lower cholesterol for treatment of hypercholesterolemia) are currently used in clinical trials. ALN-TTR02, known as Patisiran recently released clinical data that the treatment of LNPs achieved sustained serum TTR protein knockdown of 96% with a mean TTR knockdown of about 80% (www.alnylam.com). The recent report of 32 participants in Phase I had shown that ALN-PCS treatment resulted in a mean 70% reduction in circulating PCSK9 plasma protein and a mean 40% reduction in low-density lipoprotein (LDL) cholesterol from baseline relative to placebo (Fitzgerald et al., 2014).

SNALP technology from Tekmira Pharmaceuticals, Inc. is one of the most widely used lipid-based nucleic acid delivery approaches for systemic administration in clinical trials. Lipid vesicles encapsulating nucleic acids are formed instantaneously by mixing lipids

dissolved in ethanol with an aqueous solution of nucleic acids in a controlled, stepwise manner. Using this method, SNALP encapsulates nucleic acids with high efficiency (95%) in uniform LNPs, which are effective in delivering gene therapeutics especially to hepatocytes. Tekmira's lead oncology product candidate, TKM-PLK1, targets polo-like kinase 1 (PLK1), a protein involved in tumor cell proliferation and a validated oncology target. Tekmira initiated a Phase I/II clinical trial of TKM-PLK1 for patients with gastrointestinal neuroendocrine tumors, adrenocortical carcinoma, and hepatocellular carcinoma. PLK1 LNP is designed to inhibit PLK1 expression, preventing the tumor cell from completing cell division, ultimately resulting in cell cycle arrest and death of the cancer cell.

7. CONCLUSIONS

We are pleased to see that recent advances in lipid gene delivery systems have significantly improved the efficacy and the level of expression of targeted genes, the major barriers limiting the nonviral delivery method. The improved structure–activity design has increased the potential of LNPs in gene therapy in tumors as well as other disorders and diseases. However, the use of LNPs in a clinical setting may only be realistic when there is a better understanding of certain mechanisms and techniques such as: (1) the interaction of lipid vectors and gene expression, (2) the quantitation association between the vectors and gene expression level in vivo, and (3) the safety and immunogenicity profile of vectors. By overcoming these barriers, we will be closer to efficient delivery of genes by LNPs for clinical use.

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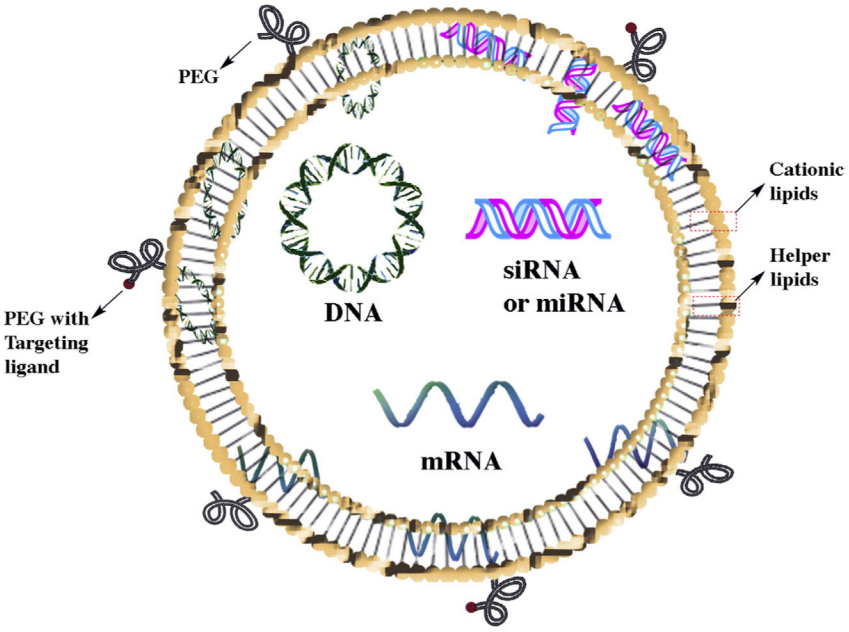

Figure 2.1.

Scheme of a lipid nanoparticle (LNP) formed by lipids (yellow), helper lipids (brown), and polyethylene glycol (PEG). Lipids condense and stabilize nucleic acids, which promote the stabilization of LNP. (See the color plate.)

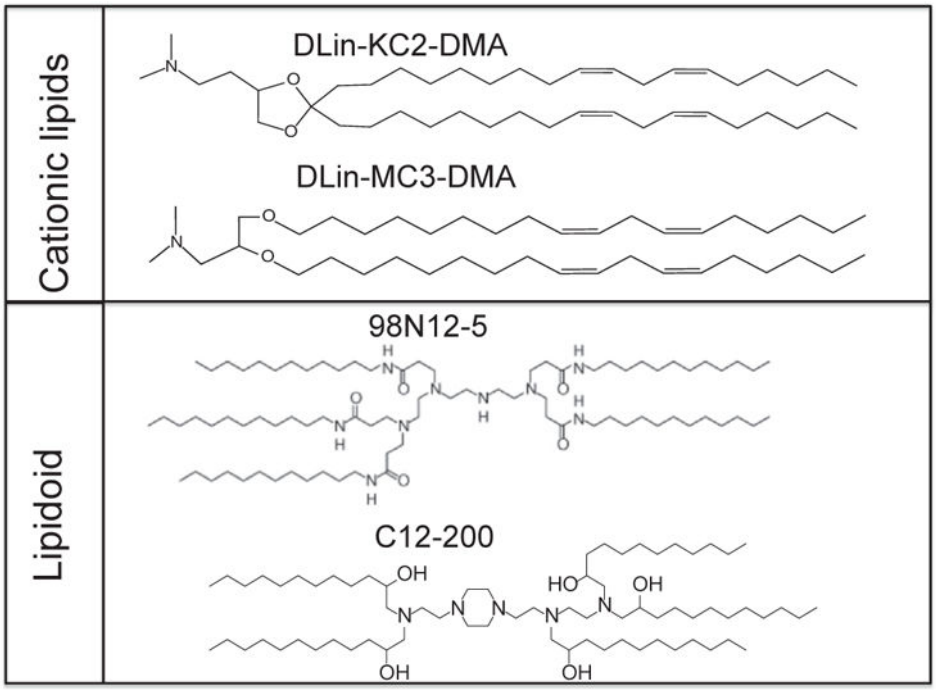

Figure 2.2.
A sampling of lipids as nonviral vectors for gene delivery.

Table 2.1

Examples of currently evaluated new LNPs for siRNA clinical trial

Therapeutic agent & company	Target	Clinical trial identifier	Disease types	Status
ALN-VSP02; Alnylam	Kinesin spindle protein; vascular endothelial growth factor	NCT00882180 NCT01158079	Solid tumor	Phase 1
ALN-TTR02; Alnylam	Transthyretin	NCT01617967 NCT01960348	Transthyretin-mediated amyloidosis	Phase 2/3
ALN-PCS02; Alnylam	Proprotein convertase subtilisin/kexin type 9	NCT01437059	Hypercholesterolemia	Phase 1
TKM-100201; Tekmira	VP24, VP35, Zaire Ebola L-polymerase	NCT01518881	Ebola virus infection	Phase 1
PRO-040201; Tekmira	ApoB	NCT00927459	Hypercholesterolemia	Phase 1
TKM 080301; Tekmira	Polo-like kinase 1	NCT01262235 NCT01437007	Neuroendocrine tumors; adrenocortical carcinoma	Phase 1/2
siRNA-EphA2-DOPC; M.D. Anderson Cancer Center	Ephrin type-A receptor 2	NCT01591356	Advanced cancers	Phase 1
Atu027; Silence Therapeutics	Protein kinase N3	NCT00938574	Advanced solid tumors	Phase 1

DOPC, dioleoylphosphatidylcholine.